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The *ompA* Signal Peptide Directed Secretion of Staphylococcal Nuclease A by *Escherichia coli**

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The hybrid pre-enzyme formed by fusion of the signal peptide of the OmpA protein, a major outer membrane protein of *Escherichia coli*, to Staphylococcal nuclease A, a protein secreted by *Staphylococcus aureus*, is translocated across the cytoplasmic membrane of *E. coli* with concomitant cleavage of the signal peptide. A DNA fragment containing the coding sequence for the *ompA* signal peptide was initially ligated to a DNA fragment containing the coding sequence for nuclease A, with a linker sequence of 33 nucleotides separating the coding sequences. When this fused gene was induced, an enzymatically active nuclease was secreted into the periplasmic space; sequential Edman degradation of this protein revealed that the *ompA* signal peptide was removed at its normal cleavage site resulting in a modified version of the nuclease having 11 extra amino acid residues attached to the amino terminus of nuclease A. The 33 nucleotides between the coding sequences for the *ompA* signal peptide and the structural gene for nuclease A were subsequently deleted by synthetic oligonucleotide-directed site-specific mutagenesis. The nuclease produced by this hybrid gene was secreted into the periplasmic space and by sequential Edman degradation was identical to nuclease A. Thus, the *ompA* signal peptide is able to direct the secretion of fused staphylococcal nuclease A, and signal peptide processing occurs at the normal cleavage site. When the hybrid gene is expressed under the control of the *lpp* promoter, nuclease A is produced to the extent of 10% of the total cellular protein.

Proteins secreted across the cytoplasmic membranes of bacteria are usually produced from a precursor molecule with an amino-terminal peptide extension (1, 2). This peptide extension, termed the signal peptide, plays an essential role in the translocation of the secreted protein across the cytoplasmic membrane. The signal peptides of Gram-negative bacteria consist of 20–25 amino acid residues; the signal peptides of Gram-positive bacteria are frequently much longer than those of Gram-negative bacteria, although all bacterial signal peptides share common structural features (3): (*a*) 1–3 basic amino acid residues in the amino-terminal region, (*b*) a hydrophobic region consisting of approximately 15 amino acid residues directly following the positively charged amino terminus, (*c*) in most signal peptides, a proline or glycine residue located within the hydrophobic core, (*d*) serine and/or threonine residue(s) located close to the carboxyl-terminal end of the hydrophobic core, and (*e*) an alanine or glycine residue at the carboxyl-terminal end (cleavage site).

Staphylococcus aureus produces an extracellular thermostable nuclease that has been structurally characterized in Anfinsen's laboratory (4) and subjected to x-ray crystallographic analysis in Cotton's laboratory (5, 6). This enzyme, termed nuclease A, consists of 149 amino acid residues and requires Ca^{2+} for catalytic activity. One of our laboratories has initiated a study of the details of catalysis and substrate binding by nuclease A using primer-directed site-specific mutagenesis as a method to generate specific mutants of active site amino acid residues. The gene for the nuclease was cloned recently by Shortle (7); this clone is poorly expressed in *Escherichia coli* and is not processed to form the mature (secreted) nuclease A. Since chemical and physical characterization of mutants requires large amounts of protein, we sought a method to produce large amounts of both wild type and mutant forms of nuclease A.

Using recombinant DNA methods, we have fused the signal peptide of the OmpA protein, a major outer membrane protein of *E. coli* (a Gram-negative organism), to the nuclease A from *S. aureus* (a Gram-positive organism). The *ompA* signal peptide is able to direct translocation of nuclease A across the cytoplasmic membrane, and the signal peptide is correctly cleaved upon accumulation of nuclease A in the periplasmic space.

EXPERIMENTAL PROCEDURES

Bacterial Strains and Plasmids—*E. coli* strains W620 *recA* (F⁻ *thi-1 pyrD36 gltA6 galK30 strA129* λ⁻ *supE44 relA recA*) (8), JA221 (*lpp⁻ hsdR⁻* Δ *trpE5 leuB6 lacY recA1/F′lacI⁹ lac⁺ pro⁺*) (9), and HB101 (*F⁻hsdS20 recA ara proA lacY galK rpsL30 xyl mtl supE*λ⁻) (10) were used. The following media were employed (11): M9 supplemented with glucose (4 mg/ml), glutamic acid (300 μg/ml), uracil (40 μg/ml), thiamine (5 μg/ml), $MgSO_4 \cdot 7H_2O$ (246 μg/ml), and ampicillin (50 μg/ml); L broth supplemented with ampicillin (50 μg/ml); and YT broth.

Plasmids pIN-III-ompA3 (12) and pFOG302 (a derivative of pFOG301 lacking the *HindIII* site in the pBR322 sequence (7)) and M13mp11 RF[1] DNA were used in the plasmid constructions and mutagenesis, respectively.

DNA Manipulations—Isolation of plasmid DNA and various routine procedures were performed as previously described (9). Restriction enzymes, T_4 DNA ligase, and polynucleotide kinase were obtained from Bethesda Research Laboratories and New England Biolabs. The Klenow fragment of DNA polymerase was from

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[1] The abbreviations used are: RF, replicative form; SDS, sodium dodecyl sulfate; IPTG, isopropyl-1-thio-β-D-galactopyranoside; bp, base pair.

Boehringer Mannheim. DNA sequencing was carried out according to Sanger *et al.* (13). Oligonucleotide-directed site-specific mutagenesis was carried out in M13mp11 (14). The oligonucleotide (34-mer; dCCGTAGCGCAGGCCGCAACTTCAACTAAAAAATT) was synthesized with an Applied Biosystems DNA synthesizer using phosphite methodology.

Expression of Hybrid Genes—Cells harboring an appropriate plasmid were grown in 10 ml of L broth. At a Klett reading of 20 with a red filter, isopropyl-1-thio-β-D-galactopyranoside (IPTG) was added to a final concentration of 2 mM. After incubation at 37 °C for 110 min, the cells were harvested by centrifugation. Cell fractionation was carried out as described previously (15) with the following modifications. After the periplasmic fraction was removed by osmotic shock (15), the resulting pellet was suspended in 500 μl of ice-cold 1 M Tris-HCl, pH 7.6, and the suspension was kept on ice for 30 min. The suspension was centrifuged in an Eppendorf microfuge for 5 min at 4 °C. One-tenth volume of ice-cold 100% (w/v) trichloroacetic acid was added to the supernatant (1 M Tris-HCl fraction), and the precipitate was collected by centrifugation and washed three times with ethanol. The pellet from the 1 M Tris-HCl wash was used to separate the cytoplasmic and membrane fractions.

For isotopic labeling experiments, *E. coli* W620 *recA* harboring pIN-III-ompA3-#98 or pONF1 was grown in 10 ml of M9 medium and labeled for 2 h at 37 °C with 100 μCi of [^{3}H]proline or [^{3}H] threonine (Amersham Corp., 40 Ci/mmol and 20 Ci/mmol, respectively). The 1 M Tris-HCl fractions were prepared as described previously and used for SDS-polyacrylamide gel electrophoresis. The radioactive nucleases were extracted from the gels and subjected to sequential Edman degradation using a JEOL Sequence Analyzer JAS-47K as previously described (16).

Authentic nuclease A was purchased from Worthington. Nuclease activity was assayed using salmon testis DNA as the substrate (17).

RESULTS

*Construction of Nuclease Expression Plasmids—Sau3A*I digestion of pFOG302, a clone containing the nuclease gene, allowed isolation of a 518-bp fragment which includes the coding region for nuclease A plus 6 extra amino acid residues forming an amino-terminal extension and 53 bp comprising a 3′-nontranslated region (7). This *Sau3A*I fragment was inserted in the *Bam*HI site of a high expression secretion vector, pIN-III-ompA, as shown in Fig. 1. With this family of cloning vectors, a foreign DNA fragment can be cloned into any one of the three reading frames at unique *Eco*RI, *Hind*III, or *Bam*HI restriction sites immediately after the coding sequence for the *ompA* signal peptide. The cloned gene is under the transcriptional control of both the *lpp* and *lac* promoters, and the expression of the gene is regulated by the *lac* repressor produced by the vector (12). For the cloning of the *Sau3A*I fragment containing the nuclease A structural gene, pIN-III-ompA with reading frame 3 (pIN-III-ompA3) was used to place the gene in reading frame with the initiation codon supplied by the vector.

The nuclease clone thus constructed, pIN-III-ompA3-#98, has an extra sequence of 33 bp between the coding sequences for the *ompA* signal peptide and nuclease A (due to the linker sequence from the vector (15 bp) and the amino-terminal extension of the nuclease (18 bp)). This extra sequence was deleted in a two-step process. First, the *Hind*III site in the linker sequence was removed by partial digestion with *Hind*III and filling in with the Klenow fragment of polymerase. After ligation and transformation of HB101, clones retaining only the *Hind*III site in the nuclease coding region (see Fig. 1) were identified by restriction analysis. The cells transformed with these plasmids were nuclease negative when tested with toluidine blue-DNA agar plates (18); this is expected for the frame shift mutation produced by the filling in of the *Hind*III site in the linker squence. Second, oligonucleotide-directed site-specific mutagenesis was used to delete the 37 bp present between the sequences coding for the *ompA* signal peptide and the nuclease A structural gene. A 422-bp *Xba*I-*Hind*III fragment containing this region was isolated from the plasmid containing the frame shift mutation and ligated into M13mp11 RF that had been digested with both *Xba*I and

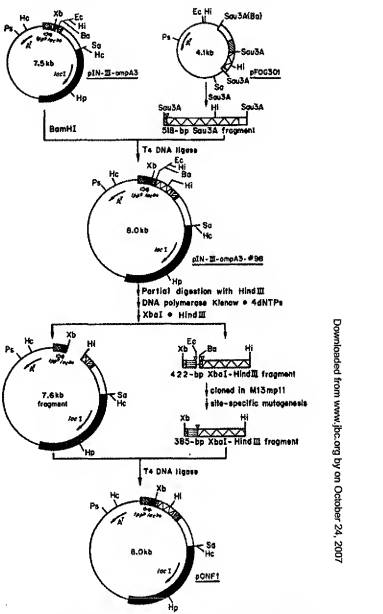

FIG. 1. **Construction of plasmids pIN-III-ompA-#98 and pONF1.** pIN-III-ompA3 is a secretion cloning vector described previously (12); the expression of a foreign gene cloned in the multirestriction site linker sequence is under the control of both the *lpp* promoter (*lpp*^{p}) and the *lac* promoter-operator (*lac*^{po}). The *lac* repressor produced from the *lacI* gene in the vector regulates the gene expression. A clone of nuclease A, pFOG302 (7), carries a 1.2-kilobase pair (kb) DNA fragment from *S. aureus* in pBR322, which includes the coding sequences for nuclease A (▨) and an amino-terminal extension (▩). The empty box (□) in the 518-bp *Sau3A*I fragment represents the 53-bp 3′-end untranslated sequence. A *triangle* indicates the position of the amino-terminal end of nuclease A. The method for the construction of pIN-III-ompA-#98 and pONF1 is described in the text. ■, the coding region for the *ompA* signal peptide; *A*^{r}, ampicillin-resistance gene (β-lactamase); *Ps*, *Pst*I; *Hc*, *Hin*cII; *Xb*, *Xba*I; *Ec*, *Eco*RI; *Hi*, *Hin*dIII; *Ba*, *Bam*HI; *Sa*, *Sal*I; *Hp*, *Hpa*I; and *Sau3A*, *Sau3A*I site.

and the nuclease A structural gene. A 422-bp *Xba*I-*Hind*III fragment containing this region was isolated from the plasmid containing the frame shift mutation and ligated into M13mp11 RF that had been digested with both *Xba*I and

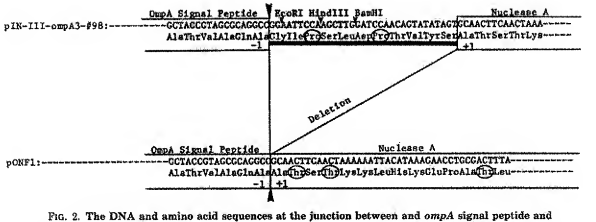

FIG. 2. **The DNA and amino acid sequences at the junction between and *ompA* signal peptide and nuclease A in pIN-III-ompA-#98 and pONF1.** The carboxyl-terminal amino acid residue (Ala) of the *ompA* signal peptide is numbered as −1 and is the 21st residue after the initiating methionine (19). The amino-terminal amino acid of nuclease A is designated as +1 (7). The *arrows* indicate the position of the cleavage site of the *ompA* signal peptide. The amino acid sequence underlined in pIN-III-ompA-#98 represents the extra sequence derived from the linker sequence (Gly-Ile-Pro-Ser-Leu) from the cloning vector, pIN-III-ompA3 (12) and a part of the amino-terminal extension (Asp-Pro-Thr-Val-Tyr-Ser) from nuclease A (7). The amino acids *circled* in the figure are those used for labeling the products for sequential Edman degradation (see Fig. 5).

*Hin*dIII. The deletion mutagenesis was performed according to a double primer method in which both the universal M13 sequencing primer and the synthetic 34-mer were used for primer extension and ligation (14). The 5′–14 bases of the 34-mer are complementary to the 3′-end of the *ompA* signal sequence (19) and the 3′–20 bases of the 34-mer are complementary to the 5′-end of the nuclease A structural gene (7). Following elongation and ligation, the M13 was digested with both *Xba*I and *Hin*dIII, and the mixture was ligated into the larger fragment of pIN-III-ompA-#98 generated by digestion with both *Xba*I and *Hin*dIII. The transformants were screened for nuclease activity with toluidine blue-DNA agar plates; 14 nuclease positive transformants were detected in a total of approximately 1600 transformants. Plasmids were isolated from 11 independent nuclease positive transformants. DNA sequence analysis of the region between the coding sequences for the *ompA* signal peptide and nuclease A allowed identification of a plasmid having the direct fusion of the coding sequences as shown in Fig. 2; this plasmid was designated pONF1 (ompA-nuclease A fusion).

Expression of Nuclease—E. coli JA221 *lpp*⁻ harboring pIN-III-ompA-#98 or pONF1 was grown for 110 min at 37 °C in L broth either in the absence or presence of 2 mM IPTG. Total cellular proteins were analyzed by SDS-polyacrylamide gel electrophoresis. A picture of a gel obtained after Coomassie Brilliant Blue staining is shown in Fig. 3. In the presence of IPTG, a new protein having a molecular weight of 18,500 was produced by pIN-III-ompA-#98 (*lane 3*), and a new protein having a molecular weight of 17,000 was produced by pONF1 (*lane 1*). These proteins were barely visible when the cells were grown in the absence of IPTG (*lane 4* for pIN-III-ompA-98 and *lane 2* for pONF1). This leaky expression of the cloned genes results from a *lac* inducing contaminant present in L broth; in M9 medium, leaky expression was not observed (data not shown). These proteins were not observed in total cellular protein obtained from cells harboring the cloning vector, pIN-III-ompA3, in the presence (*lane 5*) or absence (*lane 6*) of IPTG.

The new protein produced by cells harboring pONF1 migrates slightly slower than nuclease A obtained from a commercial source, the mobility of which is indicated by the position of the small arrow in Fig. 3; the mobility of the pONF1 encoded protein is indistinguishable from that observed for a sample of nuclease A obtained from Anfinsen's laboratory (data not shown). The molecular mass difference between the proteins produced by pIN-III-ompA-#98 and pONF1 is about 1500 daltons; this size difference is consistent with the cleavage of the signal peptide occurring at the same distance from the amino-terminal ends of both hybrid preenzymes, *e.g.* at the carboxyl-terminal end of the *ompA* signal peptide.

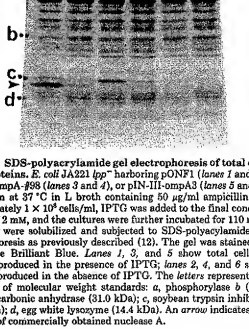

FIG. 3. **SDS-polyacrylamide gel electrophoresis of total cellular proteins.** *E. coli* JA221 *lpp*⁻ harboring pONF1 (*lanes 1* and *2*), pIN-III-ompA-#98 (*lanes 3* and *4*), or pIN-III-ompA3 (*lanes 5* and *6*) was grown at 37 °C in L broth containing 50 μg/ml ampicillin. At approximately 1×10^8 cells/ml, IPTG was added to the final concentration of 2 mM, and the cultures were further incubated for 110 min. The cells were solubilized and subjected to SDS-polyacylamide gel electrophoresis as previously described (12). The gel was stained by Coomassie Brilliant Blue. *Lanes 1*, *3*, and *5* show total cellular proteins produced in the presence of IPTG; *lanes 2*, *4*, and *6* show proteins produced in the absence of IPTG. The *letters* represent the positions of molecular weight standards: *a*, phosphorylase *b* (92.5 kDa); *b*, carbonic anhydrase (31.0 kDa); *c*, soybean trypsin inhibitor (21.5 kDa); *d*, egg white lysozyme (14.4 kDa). An *arrow* indicates the position of commercially obtained nuclease A.

The total yields of the new proteins induced by IPTG were estimated by densitometric analysis to be approximately 3 and 10% of the total cellular protein for pIN-III-ompA-#98 and pONF1, respectively; these yields were confirmed by assay of nuclease activity in the total cellular extract (data not shown). A second new protein migrating slower than the major product was observed for pONF1; this protein is the unprocessed pre-enzyme and accounts for approximately 2% of the total cellular protein. Thus, the total yield of nuclease molecules synthesized by pONF1 is about 12% of the total cellular protein.

Localization of the Cloned Gene Products—If the gene products induced by IPTG are secreted across the cytoplasmic membrane, they should be found in the periplasmic cell fraction. In the case of pIN-III-ompA-#98 (Fig. 4*A*), the nuclease was not released from the cells by a conventional osmotic shock procedure (*lane 1*). However, when the shocked cells were washed with a high salt solution (1 M Tris-HCl, pH 7.6), the nuclease was released from the cells (*lane 2*); the nuclease is the major component of this wash and comprises about 10% of the protein in this wash fraction. The washed cells were subsequently fractionated into membrane (*lane 3*) and cytoplasmic (*lane 4*) fractions; nuclease was barely detectable in these fractions.

Similarly, the nuclease produced by pONF1 was released by the high salt wash of osmotically shocked cells and accounted for 60% of the protein in the periplasmic space (Fig. 4*B*, band *y* in *lane 2*). The larger protein which was also observed upon induction of pONF1 was found in the membrane fraction (band *x* in *lane 3*). The localization of the larger induced protein in the membrane fraction suggests that this protein is the unprocessed hybrid pre-enzyme having a hydrophobic uncleaved *ompA* signal peptide. This explanation is supported by a pulse-chase experiment in which it was observed that the membrane bound protein could be chased into the periplasmic protein (data not shown). In addition, the membrane bound protein was solubilized together with other proteins located in the cytoplasmic membrane by treatment with sarcosinate (data not shown).

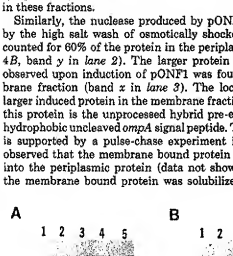

FIG. 4. **SDS-polyacrylamide gel electrophoresis of various cellular fractions of *E. coli* W620 *recA* harboring pIN-III-ompA-#98 or pONF1.** Cells were grown as described in the legend to *Fig. 3* and fractionated as described under "Experimental Procedures." *Panel A*, cells harboring pIN-III-ompA-#98; *panel B*, cells harboring pONF1. *Lane 1*, periplasmic fraction; *lane 2*, 1 M Tris-HCl fraction; *lane 3*, membrane fraction; *lane 4*, cytoplasmic fraction; *lane 5*, total cellular proteins. In *panel B*, an *arrow* marked *x* indicates the position of the unprocessed pre-enzyme and an *arrow* marked *y* indicates the position of the processed product. In both panels, the *arrow* on the *left* indicates the position of commercially obtained nuclease A. The *letters* indicate the positions of molecular weight standards: *a*, phosphorylase *b* (92.5 kDa); *b*, carbonic anhydrase (31.0 kDa); *c*, soybean trypsin inhibitor (21.5 kDa); *d*, egg white lysozyme (14.4 kDa).

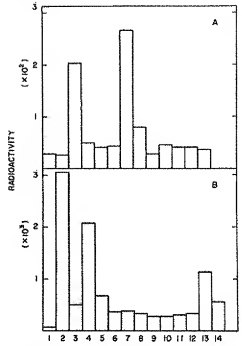

FIG. 5. **Sequential Edman degradation of the nuclease secreted across the cytoplasmic membrane.** A 20-ml culture of *E. coli* W620 *recA* harboring pIN-III-ompA-#98 or pONF1 was labeled at 37 °C for 2 h with [^{3}H]proline or [^{3}H]threonine, respectively, after induction with 2 mM IPTG. The 1 M Tris-HCl fractions were prepared and subjected to SDS-polyacrylamide gel electrophoresis. The protein corresponding to the nuclease was excised and eluted from the gel and subjected to sequential Edman degradation. *Panel A*, pIN-III-ompA-#98 labeled with [^{3}H]proline; and *panel B*, pONF1 labeled with [^{3}H]threonine.

Structural Analysis of the Cloned Gene Products—The results we have described suggest that the *ompA* signal peptide is able to direct the secretion of nuclease across the cytoplasmic membrane. Although the translocation of the nuclease should be accompanied by the cleavage of the signal peptide, the amino-terminal regions of the secreted proteins were sequenced by Edman degradation so that the cleavage sites could be identified unequivocally.

After induction of the nuclease gene in pIN-III-ompA-#98 with IPTG, the cells were labeled with [^{3}H]proline, and the secreted nuclease was isolated. Sequential Edman degradation released significant radioactivity after steps 3 and 7 (Fig. 5*A*). This result demonstrates that this nuclease is the product of cleavage of the *ompA* signal peptide at its carboxyl terminus (see Fig. 2). Similarly, after induction of the nuclease gene in pONF1 with IPTG, the cells were labeled with [^{3}H]threonine, and the secreted nuclease was isolated. Edman degradation released significant radioactivity after steps 2, 4, and 13 (Fig. 5*B*). This result also demonstrates that cleavage of the *ompA* signal peptide occurred at its carboxyl terminus and that the secreted nuclease is identical with mature nuclease A (see Fig. 2).

DISCUSSION

The data we have presented clearly demonstrate that Staphylococcal nuclease A, a Gram-positive exo-enzyme, can be secreted across the cytoplasmic membrane of *E. coli* when

the enzyme is fused to the signal peptide of the OmpA protein, a major outer membrane protein of *E. coli*. The signal peptide was cleaved at the same site in both hybrid pre-enzymes we constructed, and this site is identical to the cleavage site in the pro-OmpA protein, *i.e.* the processing of the pre-enzymes is independent of the amino acid sequences following the signal peptide. Thus, the structural determinants necessary for correct processing appear to be located on the carboxyl-terminal side of the alanine residue at position 21.

In addition to the definitive amino acid sequences data which demonstrate that cleavage of the signal peptide occurs at the normal site, our conclusion that nuclease A is translocated across the cytoplasmic membrane is supported by other observations. First, after the majority of the periplasmic proteins is released by conventional osmotic shock, the nuclease can be solubilized by a high salt wash; such treatment is not expected to release cytoplasmic proteins. The requirement for the high salt wash may be explained either by the association of the nuclease with anionic components of the periplasmic space or by aggregation due to the high protein concentration; a similar aggregation was also observed when β-lactamase was overproduced with the pIN-III-ompA vector. Second, cloning of the structural gene for nuclease A in a pIN-III vector without the *ompA* signal sequence (20) lead to production of the nuclease in the cytoplasm (data not shown). Induced cells harboring such a nuclease clone grew very poorly on L broth agar plates containing 10 mM Ca^{2+} (data not shown); this behavior is expected since the nuclease is known to require Ca^{2+} for activity (21) and can utilize the exogenously supplied metal ion to catalyze the degradation of both RNA and DNA. Induced cells harboring pIN-III-ompA-#98 could grow on L broth plates containing Ca^{2+}, thereby indicating that the nuclease produced by this plasmid did not remain in the cytoplasm but was secreted to the periplasmic space where intracellular RNA and DNA would be inaccessible.

pONF1 produces about four times as much nuclease as does pIN-III-ompA-#98. These plasmids differ only by the presence of 33 base pairs between the coding regions for the *ompA* signal peptide and nuclease A; this sequence apparently reduces the rate of translation by an unknown mechanism. At the higher level of nuclease production directed by pONF1, the cells were unable to process all of the hybrid pre-enzyme molecules; some unprocessed nuclease with the attached *ompA* signal peptide is found in the cytoplasmic membrane (Fig. 4*B*, *lane 3*). Cells harboring pONF1 were unable to form colonies on L broth agar plates containing 2 mM IPTG, and this observation can be explained by the presence of high levels of intracellular nuclease associated with the cytoplasmic membrane.

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